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Ultrasound contrast agents and process for the preparation thereof

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## ULTRASOUND CONTRAST AGENTS AND PROCESS FOR THE PREPARATION THEREOF

The present invention relates to a process for the preparation of a dry or lyophilized formulation useful for preparing a gas containing contrast agent usable in diagnostic imaging and to a process for preparing said gas containing contrast agent.

The invention also includes dry formulations prepared by this process, which may be reconstituted to form contrast agent suspensions useful in diagnostic imaging. The invention further includes suspensions of gas filled microbubbles useful in diagnostic imaging prepared using dry formulations of the invention as well as containers or two component kits containing the dry formulations of the invention.

### Background of the invention

Rapid development of ultrasound contrast agents in the recent years has generated a number of different formulations, which are useful in ultrasound imaging of organs and tissue of human or animal body. These agents are designed to be used primarily as intravenous or intra-arterial injectables in conjunction with the use of medical echographic equipment which employs for example, B-mode image formation (based on the spatial distribution of backscatter tissue properties) or Doppler signal processing (based on Continuous Wave or pulsed Doppler processing of ultrasonic echoes to determine blood or liquid flow parameters).

A class of injectable formulations useful as ultrasound contrast agents includes suspensions of gas bubbles having a diameter of few microns dispersed in an aqueous medium.

Use of suspensions of gas bubbles in carrier liquid, as efficient ultrasound reflectors is well known in the art. The development of microbubble suspensions as echopharmaceuticals for enhancement of ultrasound imaging followed early observations that rapid intravenous injections of aqueous solutions can cause dissolved gases to come out of solution by forming bubbles. Due to their substantial difference in acoustic impedance relative to blood, these intravascular gas bubbles were found to be excellent reflectors of ultrasound. The injection of suspensions of gas bubbles in a carrier liquid into the blood stream of a living organism strongly reinforces ultrasonic echography imaging, thus enhancing the visualisation of internal organs. Since imaging of organs and deep seated tissues can be crucial in establishing medical diagnosis, a lot of effort has been devoted to the development of stable suspensions of highly

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concentrated gas bubbles which at the same time would be simple to prepare and administer, would contain a minimum of inactive species and would be capable of long storage and simple distribution.

The simple dispersion of free gas bubbles in the aqueous medium is however of limited practical interest, since these bubbles are in general not stable enough to be useful as ultrasound contrast agents.

Interest has accordingly been shown in methods of stabilising gas bubbles for echography and other ultrasonic studies, for example using emulsifiers, oils, thickeners or sugars, or by entraining or encapsulating the gas or a precursor therefore in a variety of systems. These stabilized gas bubbles are generally referred to in the art as "microvesicles", and may be divided into two main categories. A first category of stabilized bubbles or microvesicles is generally referred to in the art as "microbubbles" and includes aqueous suspensions in which the bubbles of gas are bounded at the gas/liquid interface by a very thin envelope involving a surfactant (i.e. an amphiphilic material) bound at the gas to liquid interface. A second category of microvesicles is generally referred to in the art as "microballoons" or "microcapsules" and includes suspensions in which the bubbles of gas are surrounded by a solid material envelope formed of natural or synthetic polymers. Examples of microballoons and of the preparation thereof are disclosed, for instance, in European patent application EP 0458745. Another kind of ultrasound contrast agent includes suspensions of porous microparticles of polymers or other solids, which carry gas bubbles entrapped within the pores of the microparticles. The present invention is particularly concerned with contrast agents for diagnostic imaging including an aqueous suspension of gas microbubbles, i.e. microvesicles which are stabilized essentially by a layer of amphiphilic material.

Microbubbles suspensions are typically prepared by contacting powdered amphiphilic materials, e.g. freeze-dried preformed liposomes or freeze-dried or spray-dried phospholipid solutions, with air or other gas and then with aqueous carrier, agitating to generate a microbubble suspension which must then be administered shortly after its preparation.

Examples of aqueous suspension of gas microbubbles and preparation thereof can be found for instance in WO 91/15244, EP 0554 213 and WO 94/09829.

WO97/29783 discloses an alternative process for preparing gas microbubbles suspensions, comprising generating a gas microbubble dispersion in an appropriate phospholipid-containing aqueous medium and thereafter subjecting the dispersion to lyophilisation to yield a dried reconstitutable

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product. The so prepared dried products are reconstitutable in aqueous media requiring only minimal agitation. As mentioned in said document, the size of the so generated microbubbles is consistently reproducible and in practice is independent from the amount of agitation energy applied during reconstitution, being determined by the size of the microbubbles formed in the initial microbubble dispersion. The Applicant has however observed that the amount of agitation energy applied for generating the gas microbubble dispersion in the phospholipid-containing aqueous medium may be excessively high, particularly when small diameter microbubbles are to be obtained (e.g. 23000 rpm for 10 minutes, for obtaining a dispersion of bubbles having a volume mean diameter of about 3  $\mu\text{m}$ ). This high agitation energy may determine local overheating in the aqueous dispersion of microbubbles, which may in turn cause degradation of the phospholipids contained in the aqueous medium. In addition, the effects of an excessively high agitation energy are in general difficult to control and may result in an uncontrollable size distribution of the final microbubbles. Furthermore, this process involves a continuous flow of gas into the aqueous medium during the generation of microbubbles, thus requiring the use of relevant amounts of gases.

WO 94/01140 discloses a further process for preparing microvesicle suspensions reconstitutable in an aqueous medium, which comprises lyophilizing aqueous emulsions containing parenterally acceptable emulsifiers, apolar liquids and lipid-soluble or water-insoluble "structure-builders". Poloxamers and phospholipids are mentioned as parenterally acceptable emulsifiers, while mixtures of these two are employed in the working examples. Cholesterol is the preferred water-insoluble structure-builder, which is employed in the working examples. The lyophilized product is then reconstituted in water, to give aqueous suspension of gas-filled microvesicles. The gas-filled microvesicles resulting from the reconstitution step are thus defined by an envelope of different materials, including emulsifiers such as poloxamers and water-insoluble structure-builders such as cholesterol.

The process is said to result into an emulsion with particles' size lower than 4  $\mu\text{m}$ , preferably lower than 2  $\mu\text{m}$ , down to 0.5  $\mu\text{m}$ . The Applicant has however noticed that while the reconstitution step may finally result in microvesicles having a numerical mean diameter of less than 2  $\mu\text{m}$ , the corresponding size distribution of the microvesicles population is nevertheless relatively broad. In addition, the conversion step from the emulsion microparticles, obtained according to the above process, into gas microbubbles results in rather low yield.

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The Applicant has now found that a much narrower distribution of microbubbles size can be obtained if a phospholipid is used as the main parenterally acceptable emulsifier of the above emulsion and if the above process is conducted in the substantial absence of the above water-insoluble structure-builders. In addition, the substantial absence of said water-insoluble structure-builders allows to substantially increase the conversion yield from emulsion microparticles into gas microbubbles. The Applicant has further observed that the above process may result in a further narrower size distribution of microbubbles and in an increased yield if the phospholipid is the only emulsifier present in the emulsion.

The Applicant has also found that by applying a rather low agitation energy to an aqueous-organic emulsion during the process as above specified, it is possible to obtain microbubbles having a very small diameter and reduced size distribution.

#### Summary of the invention

An aspect of the present invention relates to a method for preparing a lyophilized matrix which, upon contact with an aqueous carrier liquid and a gas, is reconstitutable into a suspension of gas-filled microbubbles stabilized essentially by a phospholipid, said method comprising the steps of:

a) preparing an aqueous-organic emulsion comprising i) an aqueous medium, ii) an organic solvent substantially immiscible with water; iii) a phospholipid and iv) a lyoprotecting agent;

b) lyophilizing said emulsified mixture, to obtain a lyophilized matrix comprising said phospholipid.

Another aspect of the present invention relates to a process for preparing an injectable contrast agent comprising a liquid aqueous suspension of gas-filled microbubbles stabilized essentially by a phospholipid, which comprises the steps of:

a) preparing an aqueous-organic emulsion comprising i) an aqueous medium, ii) an organic solvent substantially immiscible with water; iii) a phospholipid and iv) a lyoprotecting agent;

b) lyophilizing said emulsion, to obtain a lyophilized matrix comprising said phospholipid.

c) contacting said lyophilized matrix with a biocompatible gas;

d) reconstituting said lyophilized matrix by dissolving it into an aqueous carrier liquid, to obtain a suspension of gas-filled microbubbles stabilized essentially by said phospholipid.

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Preferably, the step a) of preparing the emulsion comprises:

a1) preparing a solution by dispersing the phospholipid and the lyoprotective agent in the aqueous medium;

a2) admixing the obtained solution with the organic solvent;

5 a3) submitting the mixture to controlled agitation, to obtain an emulsion.

Preferably, the controlled agitation according to step a3) is obtained by using a high pressure homogenizer or more preferably a rotor-stator homogenizer.

10 A further aspect of the invention relates to an injectable contrast agent comprising a suspension of gas-filled microbubbles comprising a stabilizing layer of a phospholipid in an aqueous carrier liquid; wherein said microbubbles have a number mean diameter ( $D_N$ ) of less than  $1.70 \mu\text{m}$  and a volume median diameter ( $D_{V50}$ ) such that the  $D_{V50}/D_N$  ratio is of about 2.00 or lower.

#### Detailed description of the invention

15 As mentioned above an aspect of the present invention relates to a method for preparing a lyophilized matrix of a reconstitutable suspension of gas-filled microbubbles, said method comprising the preparation of an aqueous-organic emulsion essentially containing i) an aqueous medium, ii) an organic solvent substantially immiscible with water; iii) a phospholipid and iv) a lyoprotecting agent, and the subsequent lyophilizing of said emulsion.

20 Typically, sterile, pyrogen free water is employed as the aqueous medium in the preparation of the emulsion (to prevent as much as possible contamination in the intermediate lyophilized product). Similarly, sterile, pyrogen free saline carrier liquids may be employed.

25 As used herein the term "substantially immiscible with water" referred to the organic solvent means that, when said solvent is admixed with water, two separate phases are formed. Water immiscible solvent are generally also known in the art as apolar or non-polar solvents, as opposed to polar solvents (such as water). Water immiscible solvents are in general substantially insoluble in water.

30 For the purposes of the present invention, organic solvents suitable for being emulsified with the aqueous solvent are typically those solvents having a solubility in water of less than about 10 g/l. Preferably, the solubility of said solvent in water is of about 1.0 g/l or lower, more preferably about 0.2 g/l or lower and much more preferably about 0.01 g/l or lower. Particularly preferred

35 solvents are those having a solubility in water of 0.001 g/l or lower. Particularly insoluble organic solvents (e.g. perfluorocarbons) may have a solubility down to about  $1.0 \cdot 10^{-6}$  g/l (e.g perfluorooctane,  $1.66 \cdot 10^{-6}$  g/l).

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The organic solvent is preferably lyophilisable, i.e. said solvent has a sufficiently high vapour pressure at the lyophilization temperatures, e.g. between  $-30^{\circ}\text{C}$  and  $0^{\circ}\text{C}$ , to allow for an effective and complete  
5 evaporation/sublimation within acceptable times, e.g. 24-48 hours. Preferably, the vapour pressure of the organic solvent is higher than about 0.2 kPa at  $25^{\circ}\text{C}$ .

The organic solvent can be selected from a broad range of solvents and any chemical entity that is water-immiscible and lyophilisable, as indicated above, and being preferably liquid at room temperature ( $25^{\circ}\text{C}$ ). If a solvent having a  
10 boiling point lower than room temperature is used, the vessel containing the emulsifying mixture can advantageously be cooled below the boiling point of said solvent, e.g. down to  $5^{\circ}\text{C}$  or  $0^{\circ}\text{C}$ . As said solvent will be completely removed during the lyophilization step, no particular constraints exist except that it should not contain contaminants that cannot be removed through  
15 lyophilisation or that are not acceptable for use in an injectable composition.

Suitable organic solvents include but are not limited to alkanes, such as branched or, preferably, linear ( $\text{C}_5\text{-C}_{10}$ ) alkanes, e.g. pentane, hexane, heptane, octane, nonane, decane; alkenes, such as ( $\text{C}_5\text{-C}_{10}$ ) alkenes, e.g. 1-pentene, 2-pentene, 1-octene; cyclo-alkanes, such as ( $\text{C}_5\text{-C}_8$ )-cycloalkanes optionally  
20 substituted with one or two methyl groups, e.g. cyclopentane, cyclohexane, cyclooctane, 1-methyl-cyclohexane; aromatic hydrocarbons, such as benzene and benzene derivatives substituted by one or two methyl or ethyl groups, e.g. benzene, toluene, ethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene; alkyl ethers and ketones such as di-butyl ether and di-isopropylketone;  
25 halogenated hydrocarbons or ethers, such as chloroform, carbon tetrachloride, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane (enflurane), 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane), tetrachloro-1,1-difluoroethane, and particularly perfluorinated hydrocarbons or ethers, such as perfluoropentane, perfluorohexane, perfluoroheptane,  
30 perfluoromethylcyclohexane, perfluorooctane, perfluorononane, perfluorobenzene and perfluorodecalin, methylperfluorobutylether, methylperfluoroisobutylether, ethylperfluorobutylether, ethylperfluoroisobutylether; and mixtures thereof.

The amount of solvent is generally comprised from about 1% to about 50%  
35 by volume with respect to the amount of water used for the emulsion. Preferably said amount is from about 1% to about 20%, more preferably from about 2% to about 15% and even more preferably from about 5% to about



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10%. If desired, a mixture of two or more of the above listed organic solvents can be used, the overall amount of organic solvent in the emulsifying mixture being within the above range.

5 The term lyoprotective agent or "lyoprotectant" refers to a compound which, when included in a formulation to be lyophilized, will protect the chemical compounds from the deleterious effects of freezing and vacuumizing, such as those usually accompanying lyophilization, e.g. damage, adsorption and loss from vacuum utilized in lyophilization. In addition, after the lyophilization step, said lyoprotective agent preferably results in a solid matrix ("bulk") which supports the lyophilized phospholipid.

10 The present invention is not limited to the use of a specific lyoprotectant, and examples of suitable lyoprotectants include, but are not limited to, carbohydrates such as the saccharides, mono-, di- or poly-saccharides, e.g. glucose, galactose, fructose, sucrose, trehalose, maltose, lactose, amylose, amylopectin, cyclodextrins, dextran, inulin, soluble starch, hydroxyethyl starch (HES), sugar alcohols e.g. mannitol, sorbitol and polyglycols such as polyethyleneglycols. A substantial list of agents with lyoprotective effects is given in Acta Pharm. Technol. 34(3), pp. 129-139 (1988), the content of which is incorporated herein by reference. Said lyoprotective agents can be used singularly or as mixtures of one or more compounds.

20 Preferred lyoprotectants include mannitol and polysaccharides such as dextrans (in particular those with molecular weights above 1500 daltons), inulin, soluble starch, and hydroxyethyl starch.

25 Mixtures of mannitol or polysaccharides such as dextrans, inulin, soluble starch, hydroxyethyl starch with saccharides such as glucose, maltose, lactose, sucrose, trehalose and erythritol also provide excellent results.

30 Likewise, the present invention is not limited to any particular amount of lyoprotectant used. However the optimal weight concentration of lyoprotective agents in the emulsion prior to the lyophilisation is comprised between about 1 and about 25 %, preferably between about 2 and about 20 %, and even more preferably between about 5 and about 10 %.

A higher amount can be employed if it is also necessary to provide a desired "bulk" to the lyophilized product.

35 The lyoprotective agent is preferably added to the aqueous-organic mixture before emulsification of the same and in this case the emulsification of the aqueous-organic mixture is thus carried out in the presence of the lyoprotective agents. Alternatively, the lyoprotectant can be added to the aqueous-organic mixture after the emulsification thereof. In the first case, the

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lyoprotectant is preferably added to the aqueous medium, before admixing it with the organic solvent. If desired, it is also possible to combine the two, e.g. by adding part of the lyoprotective agent to the aqueous phase used for the preparation of the emulsion and part to the thus obtained emulsion. If desired,  
5 also cryoprotective agents, such as glycerol, can further be added to the emulsion for protecting the chemical compounds from the deleterious effects of freezing.

According to the present description and claims, the term phospholipid is intended to encompass any amphiphilic phospholipidic compound the molecules  
10 of which are capable of forming a film of material (typically in the form of a mono-molecular layer) at the gas-water boundary interface in the final microbubbles suspension. Accordingly, these material are also referred to in the art as "film-forming phospholipids". Similarly, in the emulsified mixture, these  
15 amphiphilic compounds are typically disposed at the interface between the aqueous medium and the organic solvent substantially insoluble in water, thus stabilizing the emulsified solvent microdroplets. The film formed by these compounds at the gas-water or water-solvent interface can be either continuous or discontinuous. In the latter case, the discontinuities in the film should not  
20 however be such as to impair the stability (e.g. pressure resistance, resistance to coalescence, etc.) of the suspended microbubbles or of the emulsified microdroplets, respectively. .

Amphiphilic phospholipidic compounds typically contain at least one phosphate group and at least one lipophilic long-chain hydrocarbon group.

Examples of phospholipids suitable for the process of the present invention  
25 include esters of glycerol with one or two (equal or different) molecules of fatty acids and with phosphoric acid, wherein the phosphoric acid residue is in turn bonded to a hydrophilic group, such as choline, serine, inositol, glycerol, ethanolamine, and the like groups. Fatty acids present in the phospholipids are in general long chain aliphatic acids, typically containing from 12 to 24 carbon  
30 atoms, preferably from 14 to 22, that may be saturated or may contain one or more unsaturations. Examples of suitable fatty acids are lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, oleic acid, linoleic acid, and linolenic acid. Mono esters of phospholipid are also known in the art as the "lyso" forms of the phospholipids.

35 Further examples of phospholipid are phosphatidic acids, i.e. the diesters of glycerol-phosphoric acid with fatty acids, sphingomyelins, i.e. those phosphatidylcholine analogs where the residue of glycerol diester with fatty

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acids is replaced by a ceramide chain, cardiolipins, i.e. the esters of 1,3-diphosphatidylglycerol with a fatty acid, gangliosides, cerebroside, etc.

As used herein, the term phospholipids include either naturally occurring, semisynthetic or synthetically prepared products that can be employed either singularly or as mixtures.

Examples of naturally occurring phospholipids are natural lecithins (phosphatidylcholine (PC) derivatives) such as, typically, soya bean or egg yolk lecithins.

Examples of semisynthetic phospholipids are the partially or fully hydrogenated derivatives of the naturally occurring lecithins.

Examples of synthetic phospholipids are e.g., dialkylphosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DMPC"), dipalmitoyl-phosphatidylcholine ("DPPC"), diarachidoylphosphatidylcholine ("DAPC"), distearoyl-phosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoylphosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoylphosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoylphosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl-phosphatidylcholine ("SPPC"), dioleoylphosphatidylcholine ("DOPC"), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dialkylphosphatidylglycerol ("DLPG") and its alkali metal salts, diarachidoylphosphatidylglycerol ("DAPG") and its alkali metal salts, dimyristoylphosphatidylglycerol ("DMPG") and its alkali metal salts, dipalmitoyl-phosphatidylglycerol ("DPPG") and its alkali metal salts, distearoylphosphatidylglycerol ("DSPG") and its alkali metal salts, dioleoylphosphatidylglycerol ("DOPG") and its alkali metal salts, dimyristoyl phosphatidic acid ("DMPA") and its alkali metal salts, dipalmitoyl phosphatidic acid ("DPPA") and its alkali metal salts, distearoyl phosphatidic acid ("DSPA"), diarachidoyl phosphatidic acid ("DAPA") and its alkali metal salts, dimyristoyl phosphatidyl-ethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), distearoyl phosphatidyl-ethanolamine ("DSPE"), dimyristoyl phosphatidylserine ("DMPS"), diarachidoyl phosphatidylserine ("DAPS"), dipalmitoyl phosphatidylserine ("DPPS"), distearoylphosphatidylserine ("DSPS"), dioleoylphosphatidylserine ("DOPS"), dipalmitoyl sphingomyelin ("DPSP"), and distearoyl sphingomyelin ("DSSP").

The term phospholipid further includes modified phospholipid, e.g. phospholipids where the hydrophilic group is in turn bound to another hydrophilic group. Examples of modified phospholipids are PEG (polyethylenglycol) modified phospholipids, where the hydrophilic ethanolamine

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moiety of the phospholipid is linked to a PEG molecule of variable molecular weight (from 300 to 5000 daltons).

Preferably the phospholipids employed in the process of the present invention are saturated.

5 Neutral and charged phospholipids can satisfactorily be employed in the process of the present invention, as well as mixtures thereof. As used herein and in the prior art, the term "charged" in relation with "phospholipids" means that the individual phospholipid molecules have an overall net charge, be it positive or, more frequently, negative. Preferably, blends of two or more  
10 phospholipids, at least one with a neutral charge and at least one with an overall net charge, are employed. More preferably, blends of two or more phospholipids, at least one with neutral and at least one with negative charge are employed. The amount of charged phospholipid, may vary from about 95% to about 5% by weight, with respect to the total amount of phospholipid,  
15 preferably from 80% to 20% by weight. The presence of at least minor amounts, such as 5% to 20 % by wt. with respect to the total weight of phospholipid, of a (negatively) charged phospholipid may help preventing aggregation of bubbles or emulsion droplets. It is however possible to use a single phospholipid, neutral or charged, or a blend of two or more phospholipids,  
20 all neutral or all with an overall net charge.

Preferred phospholipids are DAPC, DPPA, DSPA, DMPS, DPPS, DSPS and Ethyl-DSPC. Most preferred are DSPA, DPPS or DSPS.

Preferred mixtures of phospholipids are mixtures of DPPS with DPPC, DSPC or DAPC (from 95/5 to 5/95 w/w); mixtures of DSPA with DSPC or DAPC  
25 (from 95/5 to 5/95 w/w) , mixtures of DSPG or DPPG with DSPC or mixtures of DSPC with Ethyl-DSPC. Most preferred are mixtures of DPPS/DSPC (from 50/50 to 10/90 w/w) or DSPA/DSPC (from 50/50 to 20/80 w/w).

The amount of phospholipid is generally comprised between about 0.005 and about 1.0% by weight with respect to the total weight of the emulsified  
30 mixture. Larger amounts might of course be employed but considering that the end product is an injectable contrast agent, it is preferred not to use excess of additives unless strictly necessary to provide for a stable and suitable product. In general, by using an amount of phospholipid larger than that indicated as the upper limit of the above range, essentially no or a very negligible improvement  
35 is observed in terms of bubble population, bubble size distribution, and bubble stability. Typically, higher amounts of phospholipid are required when higher volumes of organic solvent are used. Thus, when the volume of organic solvent amounts to about 50% the volume of the water phase, an amount of about 1%

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w/w of phospholipid can advantageously be added to the emulsion. Preferably the amount of phospholipid is comprised between 0.01 and 1.0% by weight with respect to the total weight of the emulsified mixture and more preferably between about 0.05% and 0.5% by weight.

5 As mentioned before, the microbubbles produced according to the process of the invention are stabilized essentially by a phospholipid, as above defined. In particular, the envelope surrounding the gas filled microbubbles is formed for at least 80%, preferably for at least 90% and more preferably for substantially its totality of a phospholipid material as above defined.

10 If it is desired to obtain "targeted" ultrasound contrast agents, i.e. contrast agents containing microbubbles that could selectively bind to a specific site after *in vitro* or *in vivo* administration, according to the process of the present invention it is also possible to start directly from a phospholipid at least part of which has been modified by the introduction of a suitably selected  
15 targeting ligand or alternatively, and preferably, starting from phospholipid at least part of which contain a possibly protected reactive group capable of being coupled at a later stage with the suitably selected targeting ligand containing a complementary reactive function (e.g. avidin-biotin link).

20 Therefore, in this specific context, the term "phospholipid" is intended to encompass both modified and unmodified phospholipids, thus including phospholipids modified by linking a targeting ligand or a protective reactive group to the amphiphilic molecule of the phospholipid.

25 As used herein the term "targeting ligand" refers to any material or substance, which may promote targeting of tissues and/or receptors *in vivo* with the compositions of the present invention. The targeting ligand may be synthetic, semi-synthetic, or naturally-occurring. Materials or substances which may serve as targeting ligands include, for example, but are not limited to proteins, including antibodies, antibody fragments, receptor molecules, receptor  
30 binding molecules, glycoproteins and lectins, peptides, peptidomimetics, saccharides, including mono and polysaccharides, vitamins, steroids, steroid analogs, hormones, cofactors, bioactive agents, and genetic material, including nucleosides, nucleotides and polynucleotides.

Examples of suitable targets and targeting ligands are disclosed, for instance, in US patent no. 6,139,819, which is herein incorporated by reference.

35 In one preferred embodiment the targeting ligands can be bound to the amphiphilic molecules through a covalent bond.

In such a case the specific reactive moiety that needs to be present in the phospholipid or lipid molecule when a targeting amphiphilic molecule is

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desired, will depend on the particular targeting ligand to be coupled thereto. As an example, if the targeting ligand can be linked to the amphiphilic molecule through an amino group, suitable reactive moieties for the amphiphilic molecule may be isothiocyanate groups (that will form a thiourea bond), reactive esters (to form an amide bond), aldehyde groups (for the formation of an imine bond to be reduced to an alkylamine bond), etc.; if the targeting ligand can be linked to the amphiphilic molecule through a thiol group, suitable complementary reactive moieties for the amphiphilic molecule include haloacetyl derivatives or maleimides (to form a thioether bond); and if the targeting ligand can be linked to the amphiphilic molecule through a carboxylic group, suitable reactive moieties for the amphiphilic molecule might be amines and hydrazides (to form amide or alkylamide bonds).

As indicated above, in a preferred embodiment, when a contrast agent containing targeted microbubbles is desired, at least part of the starting phospholipid will contain a suitable reactive moiety and the targeting ligand containing the complementary functionality will be linked thereto either at any step before the lyophilization, by adding the targeting ligand containing the complementary functionality the phase containing the functionalised phospholipids/lipids, either before, during or after the generation of the emulsion, or just before the reconstitution step. In this latter case it would be possible to fully exploit the flexibility of the system as the microbubbles containing at least part of the film-forming phospholipids, or of the associated lipids, suitably functionalised, might then be bound to any desired targeting ligand, sharing the same reactive complementary group.

Not necessarily however the targeting ligand needs to be bound to the amphiphilic molecules through a covalent bond. The targeting ligands may also be suitably associated to the microbubbles via physical and/or electrostatic types of interactions. As an example, a functional moiety having a high affinity and selectivity for a complementary moiety can be introduced into the phospholipid molecule, while the complementary moiety will be linked to the targeting ligand. For instance, an avidin (or streptavidin) moiety (having high affinity for biotin) can be covalently linked to a microbubble stabilizing phospholipid while the complementary biotin moiety can be incorporated into a suitable targeting ligand, e.g. a peptide or an antibody. The biotin-labelled targeting ligand will thus be associated to the avidin-labelled microbubble by means of the avidin-biotin coupling system. Examples of biotin/avidin labelling of phospholipids and peptides are also disclosed in the above cited US 6,139,819.. Alternatively, van der Waal's interactions, electrostatic interactions

and other association processes may associate or bind the targeting ligand to the amphiphilic molecules.

5 Examples of suitable targets are, for instance, fibrin and the GPIIb/IIIa binding receptor on activated platelets. Fibrin and platelets are in fact generally present in "thrombi", i.e. coagula which may form in the blood stream and cause a vascular obstruction. Other examples of important targets include  
10 receptors in vulnerable plaques and tumor specific receptors. Suitable binding peptides are disclosed, for instance, in the above cited US 6,139,819. Binding peptides specific for fibrin-targeting are also disclosed, for instance, in International patent application WO 02/055544, which is herein incorporated by reference.

The emulsifying step a) of the process of the present invention can be carried out by submitting the aqueous medium and the core solvent in the presence of at least one phospholipid, as indicated above, to any appropriate  
15 emulsion-generating technique known in the art, such as, for instance, sonication, shaking, high pressure homogenization, micromixing, membrane emulsification, high speed stirring or high shear mixing, e.g. using a rotor-stator homogenizer. Preferably, a rotor-stator homogenizer is employed, such as Polytron® PT3000. The agitation speed of the rotor-stator homogenizer can be  
20 selected depending from the components of the emulsion, the volume of the emulsion and of the diameter vessel containing the emulsion and the desired final diameter of the microdroplets of solvent in the emulsion. In general, it has been observed that, when using a rotor-stator homogenizer having a probe of about 3 cm diameter immersed in a 50-80 ml mixture contained in 3.5-5 cm  
25 diameter beaker, an agitation speed of about 8000 rpm is typically sufficient to obtain microdroplets having a mean numerical diameter sufficiently reduced to result, after lyophilization and reconstitution of the lyophilized matrix, in gas-filled microbubbles having a diameter of less than about 1.8 µm. By increasing the agitation speed at about 12000 rpm, it is in general possible to obtain gas-filled microbubbles having a number mean diameter of less than about 1.5 µm,  
30 while with an agitation speed of about 14000-15000 rpm, gas-filled microbubbles having a number mean diameter of about 1.0 µm or less can generally be obtained. In general it has been observed that by increasing the agitation speed above about 18000 rpm, slight further reduction of  
35 microbubbles size is obtained.

The organic solvent can be introduced gradually during the emulsification step or at once before starting the emulsification step. Alternatively the aqueous medium may be gradually added to the water immiscible solvent during the

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emulsification step or at once before starting the emulsification step. Preferably, the phospholipid is dissolved in the aqueous medium before this latter is admixed with the organic solvent. Alternatively, the phospholipid can be dissolved in the organic solvent or it may be separately added the aqueous-  
5 organic mixture before or during the emulsification step.

In a preferred embodiment, the emulsification of step a) is conveniently carried out at room temperature, e.g. at a temperature of  $25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ , but may also be carried out at higher temperatures, for instance  $50^{\circ}\text{C}$ - $60^{\circ}\text{C}$  (in the case of core solvents with high boiling points) or at lower temperature, for instance  
10  $0^{\circ}\text{C}$ - $10^{\circ}\text{C}$  (in the case of core solvents with boiling points close to room temperature). The temperature is in any case kept lower than the boiling temperature of the organic solvent, preferably at least  $5^{\circ}\text{C}$  below said temperature, more preferably at least  $10^{\circ}\text{C}$  below. In addition, it is preferable to avoid prolonged exposure of the mixture at high temperatures (e.g.  $80^{\circ}\text{C}$ -  
15  $90^{\circ}\text{C}$ ), in order to avoid possible degradations of phospholipids.

If necessary, the aqueous medium containing the phospholipids can be subjected to controlled heating, in order to facilitate the dispersion thereof. For instance, the phospholipid containing aqueous solution can be heated at about  $70^{\circ}\text{C}$  for about 15 minutes and then allowed to cool at the temperature at which  
20 the emulsion step is then carried out.

Whilst according to a preferred embodiment of the present invention the emulsion of step a) is prepared by using a phospholipid as the only surfactant of the aqueous organic mixture, limited amounts of other amphiphilic materials can nevertheless be added to the mixture. The amount of said additional amphiphilic  
25 is preferably not higher than about 20% by weight with respect to the total weight of amphiphilic material, more preferably not higher than 10% by weight, down to an amount of about 0.1%.

These further amphiphilic materials shall preferably have a rather low HLB (hydrophilic-lipophilic balance) value, e.g. lower than about 15, more preferably  
30 lower than about 12, much more preferably lower than about 10. As known in the art, the HLB is indicative of the polarity and of the emulsification behavior of a substance and is related to the balance between the hydrophilic and lipophilic portions of the molecule. In particular, the HLB is a number between 0 and 40 assigned to emulsifying agents and substances which are emulsified; higher  
35 values of HLB identify more hydrophilic surfactants, while more lipophilic surfactants have generally lower HLB values. HLB values of surfactants are generally given by the manufacturers or may be empirically calculated from the chemical formula of the surfactant, as described for instance in "Surfactants and



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Polymers Drug Delivery", Martin Malmsten, Marcel Dekker Ed. 2002, pp. 56-60). Preferably, said amphiphilic material is a fatty acid, such as those previously mentioned, a glycolipid or mixtures thereof. Small amounts of fatty acids and lyso forms of the phospholipids may also form as degradation products of the original phospholipid products, e.g. as a consequence of heating the emulsion

The aqueous medium may, if desired, further contain one or more excipients.

As used herein, the term "excipient" refers to any additive useful in the present invention, such as those additives employed to increase the stability of the emulsion or of the lyophilisate intermediate and/or to provide for pharmaceutically acceptable and stable final compositions.

Exemplary excipients in this regard are, for instance, viscosity enhancers and/or solubility aids for the phospholipids.

Viscosity enhancers and solubility aids that may suitably be employed are for example mono- or polysaccharides, such as glucose, lactose, saccharose, and dextrans, aliphatic alcohols, such as isopropyl alcohol and butyl alcohol, polyols such as glycerol, 1,2-propanediol, and the like agents. In general however we have found that it is unnecessary to incorporate additives such as viscosity enhancers, which are commonly employed in many existing contrast agent formulations, into the contrast agents of the present invention. This is a further advantage of the present invention as the number of components administered to the body of a subject is kept to a minimum and the viscosity of the contrast agents is maintained as low as possible.

As mentioned before, the Applicant has found substantially unnecessary if not disadvantageous to add water-insoluble builders, such as cholesterol, to the emulsifying mixture. As a matter of fact, it has been observed that an amount of 0.05% (w/w), with respect to the total weight of the emulsifying mixture, of cholesterol dramatically reduces the conversion yield from microdroplets into gas-filled microvesicles, further resulting in a broad-dispersion of the vesicles' size. Thus, the emulsifying aqueous-organic mixture is substantially free of any water-insoluble component (apart of course the organic solvent). In particular, if present, its amount is lower than 0.050%, preferably lower than about 0.030% by weight with respect to the total weight of the emulsion.

If desired, the obtained emulsion may optionally be submitted to a sterilization step. For instance the emulsion can be advantageously heated at about 120°C for about 15 minutes.

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Emulsions produced according to step a), optionally sterilized, may advantageously be subjected to one or more washing steps, prior to the lyophilization of step b), in order to remove excess of phospholipids in the aqueous phase (not associated to the emulsion) and separate and remove optional additives such as viscosity enhancers and solubility aids, as well as unwanted material such as colloidal particles, and undersized and/or oversized emulsion droplets. Such washing may be effected in *per se* known manner, the emulsion being separated using techniques such as flotation, centrifugation, cross flow filtration.

If washing steps are foreseen, and if a lyoprotective agent was present in the original aqueous phase prior to the generation of the emulsion, said washing steps can be performed with aqueous solutions containing one or more lyoprotective agents to somehow replace the amount of lyoprotective agents removed with the washings. On the other side, if no lyoprotectant was present in the emulsified aqueous-organic mixture, the formed emulsion can be washed with a lyoprotectant-containing aqueous solution, in order to introduce the lyoprotectant into the emulsified mixture or, alternatively, the lyoprotectant can be added after the washing steps, prior to lyophilisation.

If desired, the emulsion (either as such or after the washing step) can be subjected to a microfiltration step before lyophilization, in order to further reduce the amount of large size microbubbles in the final reconstituted suspension. During microfiltration, e.g. with a 5  $\mu\text{m}$  or 3  $\mu\text{m}$  filter, large size microdroplets are in fact retained by the filter and separated from the rest of the small size microdroplets, thus preventing the formation of large size microbubbles upon reconstitution of the lyophilized material. Microfiltration can be accomplished according to conventional techniques such as positive filtration, vacuum filtration or in-line filtration. Membranes of filtration can be Nylon, glass fiber, cellulose, paper, polycarbonate or polyester (Nuclepore®) membranes.

Lyophilization of the emulsion according to step b) may be carried out by initially freezing it and thereafter lyophilising the frozen emulsion, by *per se* generally known methods and devices. Since the dried, lyophilized, product will normally be reconstituted by addition of a carrier liquid prior to administration, the emulsion may advantageously be filled into sealable vials prior to lyophilisation so as to give vials each containing an appropriate amount, e.g. a single dosage unit, of lyophilised dried product for reconstitution into an injectable form. By lyophilising the emulsion in individual vials rather than in bulk, handling of the delicate honeycomb-like structure of the lyophilised product and the risk of at least partially degrading this structure are avoided.

Following lyophilisation, the vacuum may be broken in the lyophilizer by introducing the gas desired to be present in the microbubbles in the ultimately formulated contrast agent. This will allow to fill the headspace of the vials with the desired gas and then seal the vials with an appropriate closure.

5 Alternatively, the vial can be kept under vacuum and sealed, while the gas is added at a later stage, e.g. just before administration, for instance when the gas is a radioactive or hyperpolarized gas.

The so obtained lyophilized product in the presence of the suitable gas can thus be stably stored for several months before being reconstituted by  
10 dissolving it into an aqueous carrier liquid, to obtain a suspension of gas-filled microbubbles.

Suitable gases for forming the gas-filled microbubbles are those biocompatible gases known and generally employed in the art. The term "gas" as used herein includes any substance (including mixtures) substantially or  
15 completely in gaseous (including vapor) form at the normal human body temperature of 37 °C. Compounds which at the temperature of 37°C are liquid are thus used in admixture with other gaseous compounds, to obtain a mixture which is in the gaseous phase at 37°C. Suitable gases comprise, for example, air; nitrogen; oxygen; carbon dioxide; hydrogen; nitrous oxide; an inert gas  
20 such as helium, argon, xenon or krypton, including hyperpolarized gases such as hyperpolarized helium and hyperpolarized xenon and radioactive gases such as <sup>133</sup>Xe and <sup>81</sup>Kr; a sulphur fluoride such as sulphur hexafluoride, or trifluoromethylsulphur pentafluoride; a low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms), for example a (C<sub>1</sub>-C<sub>7</sub>) alkane such as  
25 methane, ethane, propane, butane, iso-butane, pentane or isopentane, a (C<sub>4</sub>-C<sub>7</sub>) cycloalkane such as cyclobutane or cyclopentane, a (C<sub>2</sub>-C<sub>7</sub>) alkene such as propene or a butene, or a (C<sub>2</sub>-C<sub>7</sub>) alkyne such as acetylene; an ether; a ketone; an ester; a halogenated low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms); or a mixture of any of the foregoing. At least some of the  
30 halogen atoms in halogenated gases are advantageously fluorine atoms. Examples of biocompatible halogenated hydrocarbon gases are bromochlorodifluoro-methane, chlorodifluoromethane, dichlorodifluoro-methane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, and dichlorotetrafluoroethane. According to a most preferred embodiment, the gas is  
35 a perfluorinated hydrocarbon. Examples of perfluorocarbons are perfluoroalkanes such as perfluoromethane, perfluoroethane, perfluoropropanes, perfluorobutanes, (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-iso-butane), perfluoropentanes, perfluorohexanes and

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perfluoroheptanes; perfluoroalkenes such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-ene) and perfluorobutadiene; perfluoroalkynes such as perfluorobut-2-yne; and perfluorocycloalkanes such as perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethylcyclo-butanes, perfluorocyclopentane, perfluoromethylcyclopentane, perfluorodimethylcyclo-pentanes, perfluorocyclohexane, perfluoromethylcyclohexane and perfluorocycloheptane. Other halogenated gases include fluorinated, e.g. perfluorinated, ketones such as perfluoroacetone and fluorinated, e.g. perfluorinated, ethers such as perfluorodiethyl ether.

In the contrast agents of the invention it may be advantageous to employ fluorinated gases such as sulphur fluorides or fluorocarbons, e.g. perfluorocarbons such as perfluoropropane or perfluorobutane, which are known to form particularly stable microbubble suspensions. Examples of gases forming particularly stable microbubble suspension are disclosed, for instance, in EP 0554 213, which is herein incorporated by reference.

It may also be advantageous to use a gas mixture comprising e.g. a conventional gas, such as nitrogen, air or carbon dioxide and a gas forming a stable microbubble suspension, such as sulphur hexafluoride or a perfluorocarbon as indicated above. Examples of suitable gas mixtures can be found, for instance, in WO 94/09829, which is herein incorporated by reference.

The lyophilized composition in contact with the gas can then be very easily reconstituted by the addition of an appropriate sterile aqueous injectable and physiologically acceptable carrier liquid such as sterile pyrogen-free water for injection, an aqueous solution such as saline (which may advantageously be balanced so that the final product for injection is not hypotonic), or an aqueous solution of one or more tonicity-adjusting substances such as salts (e.g. of plasma cations with physiologically tolerable counterions), or sugars, sugar alcohols, glycols and other non-ionic polyol materials (e.g. glucose, sucrose, sorbitol, mannitol, glycerol, polyethylene glycols, propylene glycols and the like), requiring only minimal agitation such as may, for example, be provided by gentle hand-shaking. As observed by the Applicant, the so obtained reconstituted microbubbles have generally a number mean diameter which is slightly lower than the number mean diameter measured for the microdroplets of the emulsion. The mean number diameter of the microbubbles is in general from about 60% to about 90% of the mean number diameter of the emulsion's microdroplets. In most cases, a mean number diameter of the microbubbles of

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about 70-75% of the mean number diameter of the microdroplets has been observed;

Where the dried product is contained in a vial, this is conveniently sealed with a septum through which the carrier liquid may be injected using an optionally pre-filled syringe; alternatively the dried product and carrier liquid may be supplied together in a dual chamber device such as a dual chamber syringe. It may be advantageous to mix or gently shake the product following reconstitution. However, as noted above, in the stabilized contrast agents according to the invention the size of the gas microbubbles may be substantially independent of the amount of agitation energy applied to the reconstituted dried product. Accordingly no more than gentle hand-shaking may be required to give reproducible products with consistent microbubble size.

The microbubble suspensions generated upon reconstitution in water or an aqueous solution may be stable for at least 12 hours, thus permitting considerable flexibility as to when the dried product is reconstituted prior to injection.

Unless it contains a hyperpolarized gas, known to require special storage conditions, the lyophilised residue may be stored and transported without need of temperature control of its environment and in particular it may be supplied to hospitals and physicians for on site formulation into a ready-to-use administrable suspension without requiring such users to have special storage facilities.

Preferably in such a case it can be supplied in the form of a two component kit.

Said two component kit can include two separate containers or a dual-chamber container. In the former case preferably the container is a conventional septum-sealed vial, wherein the vial containing the lyophilized residue of step b) is sealed with a septum through which the carrier liquid may be injected using an optionally prefilled syringe. In such a case the syringe used as the container of the second component is also used then for injecting the contrast agent. In the latter case, preferably the dual-chamber container is a dual-chamber syringe and once the lyophilisate has been reconstituted and then suitably mixed or gently shaken, the container can be used directly for injecting the contrast agent. In both cases means for directing or permitting application of sufficient bubble forming energy into the contents of the container are provided. However, as noted above, in the stabilised contrast agents according to the invention the size of the gas microbubbles is substantially independent of the amount of agitation energy applied to the reconstituted dried product. Accordingly no more

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than gentle hand shaking is generally required to give reproducible products with consistent microbubble size.

It can be appreciated by one ordinary skilled in the art that other two-chamber reconstitution systems capable of combining the dried powder with the aqueous solution in a sterile manner are also within the scope of the present invention. In such systems, it is particularly advantageous if the aqueous phase can be interposed between the water-insoluble gas and the environment, to increase shelf life of the product. Where a material necessary for forming the contrast agent is not already present in the container (e.g. a targeting ligand to be linked to the phospholipid during reconstitution), it can be packaged with the other components of the kit, preferably in a form or container adapted to facilitate ready combination with the other components of the kit.

No specific containers, vial or connection systems are required; the present invention may use conventional containers, vials and adapters. The only requirement is a good seal between the stopper and the container. The quality of the seal, therefore, becomes a matter of primary concern; any degradation of seal integrity could allow undesirable substances to enter the vial. In addition to assuring sterility, vacuum retention is essential for products stoppered at ambient or reduced pressures to assure safe and proper reconstitution. As to the stopper, it may be a compound or multicomponent formulation based on an elastomer, such as poly(isobutylene) or butyl rubber.

The contrast agents obtainable by the process of the present invention may be used in a variety of diagnostic imaging techniques, including in particular ultrasound and Magnetic Resonance. Possible other diagnostic imaging applications include scintigraphy, light imaging, and X-ray imaging, including X-ray phase imaging..

Their use in diagnostic ultrasound imaging and in MR imaging, e.g. as susceptibility contrast agents and as hyperpolarized gas bubbles, constitute preferred features of the invention. A variety of imaging techniques may be employed in ultrasound applications, for example including fundamental and harmonic B-mode imaging, pulse or phase inversion imaging and fundamental and harmonic Doppler imaging; if desired three-dimensional imaging techniques may be used.

In vivo ultrasound tests in rabbits, dogs and pigs have shown that contrast agents according to the invention may produce an increase in backscattered signal intensity from the myocardium of 15-25 dB following intravenous injection of doses as low as 0.001 ml/kg body weight. Signals may be observed at even lower doses using more sensitive techniques such as color Doppler or

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power pulse inversion. At these low doses, attenuation in blood-filled compartments such as the heart chambers has been found to be sufficiently low to permit visualization of regions of interest in the myocardial vasculature. Tests have also shown such intravenously injected contrast agents to be distributed  
5 throughout the whole blood pool, thereby enhancing the echogenicity of all vascularised tissues, and to be recirculated. They have also been found useful as general Doppler signal enhancement aids, and may additionally be useful in ultrasound-computed tomography and in physiologically triggered or intermittent imaging.

10 For ultrasound applications such as echocardiography, in order to permit free passage through the pulmonary system and to achieve resonance with the preferred imaging frequencies of about 0.1-15 MHz, microbubbles having an average size of 0.1-10  $\mu\text{m}$ , e.g. 0.5-7  $\mu\text{m}$  are generally employed. As described above, contrast agents according to the invention may be produced with a very  
15 narrow size distribution for the microbubble dispersion within the range preferred for echocardiography, thereby greatly enhancing their echogenicity as well as their safety in vivo, and rendering the contrast agents of particular advantage in applications such as blood pressure measurements, blood flow tracing and ultrasound tomography.

20 In ultrasound applications the contrast agents of the invention may, for example, be administered in doses such that the amount of phospholipid injected is in the range 0.1-200  $\mu\text{g/kg}$  body weight, typically 10-200  $\mu\text{g/kg}$  in the absence of a washing step for the emulsion and 0.1-30  $\mu\text{g/kg}$  if the emulsion has been washed prior to lyophilisation. It will be appreciated that the  
25 use of such low levels of phospholipid is of substantial advantage in minimising possible toxic side effects. Furthermore, the low levels of phospholipids present in effective doses may permit dosage increases to prolong observation times without adverse effects.

30 According to a preferred embodiment of the invention, the process of the invention allows to obtain small diameter gas-filled microbubbles showing an extremely narrow size distribution. Thus, by suitably selecting the components of the mixture and in particular the amount of agitation energy applied during the emulsion of the aqueous-organic mixture, it is possible to obtain gas-filled microbubbles with the desired numerical mean diameter and size distribution.

35 In particular, by exploiting the process according to the present invention it is possible to obtain contrast agents comprising phospholipid-stabilized small-sized gas microbubbles characterized by having relatively small mean dimensions and a particularly useful narrow and controlled size distribution.

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As known by those skilled in the art, particles' dimensions and size distribution can be characterized by a number of parameters, the most frequently used being the mean diameter in number  $D_N$ , the median diameter in number  $D_{N50}$ , the mean diameter in volume  $D_V$  and the median diameter in volume  $D_{V50}$ . While diameters in number provide an indication of the mean number dimension of the bubbles, the diameter in volume provides information on how the total volume of gas entrapped in the microbubbles is distributed among the bubble population. As the presence of very few large volume microbubbles in a population of otherwise small volume microbubbles may cause the corresponding  $D_V$  value of the set of microbubbles to be shifted towards high values, the Applicant has found more convenient to use the  $D_{V50}$  value for evaluating the distribution of a microbubbles' population. The  $D_{V50}$  value is in fact a calculated value indicating that half of the total of bubble-entrapped gas is present in bubbles having a diameter lower than  $D_{V50}$ ; this allows to reduce the effect of accidentally formed large volume microbubbles in the evaluation of the size distribution. Clearly mono-sized bubbles show identical  $D_N$ ,  $D_{N50}$ ,  $D_V$  and  $D_{V50}$  values. On the other side, an increasing broadening of microbubbles' distribution will result in a larger difference between these various values and, accordingly, the respective ratio thereof will increase. Thus for example bubble populations containing primarily small bubbles (i.e. bubbles with a diameter around 2  $\mu\text{m}$ ) with nevertheless a small percentage of large bubbles (for instance bubbles with a diameter above 8  $\mu\text{m}$ ) will show high  $D_V$  or  $D_{V50}$  values compared to  $D_N$ , with correspondingly higher  $D_V/D_N$  or  $D_{V50}/D_N$  ratios.

The process of the present invention has thus been found particularly suitable to prepare microbubbles having a mean diameter in number ( $D_N$ ) of less than 1.70  $\mu\text{m}$  and a median diameter in volume ( $D_{V50}$ ) such that the  $D_{V50}/D_N$  ratio is of about 2.30 or lower, preferably lower than 2.10. Preferably said  $D_N$  value is of 1.60  $\mu\text{m}$  or lower, more preferably of 1.50  $\mu\text{m}$  or lower, much more preferably of 1.30  $\mu\text{m}$  or lower. Microbubbles with lower values of  $D_N$ , e.g. of about 1  $\mu\text{m}$ , or even lower, e.g. 0.85  $\mu\text{m}$  and down to 0.80  $\mu\text{m}$ , can easily be obtained with the process of the invention. The  $D_{V50}/D_N$  ratio is preferably of about 1.80 or lower, more preferably of about 1.60 or lower, much more preferably of about 1.50 or lower. Microbubbles with lower values of the  $D_{V50}/D_N$  ratio, e.g. 1.20, and even lower, e.g. 1.05, can easily be obtained.

Furthermore, in the suspensions of small size narrowly-distributed microbubbles obtainable according to the process of the invention, it has been observed that the amount of microbubbles with a diameter larger than 3  $\mu\text{m}$ , in



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particular for microbubbles having a  $D_N$  lower than about  $1.5 \mu\text{m}$  and a  $D_{V50}/D_N$  ratio of less than about 2.00, is typically lower than about 3% with respect to the total number of microbubbles in the suspension, preferably lower than about 2%, more preferably lower than about 1%. The concentration of microbubbles in the reconstituted suspension is in general of at least  $1 \times 10^8$  particles per milliliter, preferably of at least  $1 \times 10^9$  particles per milliliter.

The above values of  $D_{V50}$ ,  $D_N$  and number of microbubbles are referred to a measurement made by using a Coulter Counter Mark II apparatus fitted with a  $30 \mu\text{m}$  aperture, with a measuring range of 0.7 to  $20 \mu\text{m}$ .

This specific category of contrast agents are particularly valuable in ultrasound imaging, in the specific for imaging techniques relying on non-linear scattering of microbubbles, as explained below.

A number of the most recent ultrasound contrast-imaging methods exploit the nonlinear scattering characteristics of ultrasound contrast agents. From the literature (e.g. Eatock et al., Journal of the Acoustical Society of America, vol.77(5), pp1692-1701, 1985) it is known that nonlinear scattering is significant only for microbubbles which are smaller than, or close to, resonance size, and mainly for microbubbles smaller than half the resonance size. "Half the resonance size" is the size of a microbubble with a resonance frequency that equals twice the center frequency of the transmitted ultrasound wave (which for particular applications may be of up to about 60 Mhz). When imaging a volume containing a microbubble-based ultrasound contrast agent, the detectability of the microbubble echoes against tissue echoes is enhanced by the level of nonlinear scattering by the microbubbles, and decreased by the attenuation caused by the microbubbles located between the probe and the region of interest. Attenuation along the transmit path reduces the ultrasound-energy available for generating nonlinear bubble-response; attenuation along the receive path removes echo-energy able to reach the ultrasound probe. In the case of a suspension comprising a wide range of microbubble sizes, the microbubbles at resonance size, and larger than resonance size, mainly contribute to transmit-receive attenuation, without contributing in an efficient way to the nonlinear echo signals. Therefore, the overall acoustic response for nonlinear imaging greatly benefits from the use of a calibrated set of microbubbles having a narrow size distribution and a mean size close to half the resonance size or smaller.

A yet still further aspect of the present invention thus relates to a method of diagnostic imaging which comprises administering to a subject a contrast-enhancing amount of a contrast agent comprising gas-filled microbubbles with

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the size and size distribution as above specified and imaging at least a part of said subject by diagnostic imaging. In particular, said diagnostic imaging includes non-linear scattering imaging. According to this method, said subject is a vertebrate and said contrast agent is introduced into the vasculature or into a body cavity of said vertebrate. Said contrast agent can be supplied as a kit, such as those previously described, comprising the lyophilized product in contact with the gas and an aqueous medium for reconstitution.

The following non-limitative examples are given for better illustrating the invention.

#### Examples

The following materials have been employed in the following examples.

#### PHOSPHOLIPIDS:

- DPPS: dipalmitoilphosphatidylserine (1,2-dipalmitoyl-sn-glycero-3-phosphoserine), from Genzyme
- DPPG: dipalmitoylphosphatidylglycerol (1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (sodium salt)), from Genzyme
- DSPA: distearoylphosphatidic acid (1,2-distearoyl-sn-glycero-3-phosphate (sodium salt)), from Sygena
- DSPG: distearoylphosphatidylglycerol (1,2-distearoyl-sn-glycero-3-phosphoglycerol (sodium salt)), from Sygena
- DSPC: distearoylphosphatidylcholine (1,2-distearoyl-sn-glycero-3-phosphocholin). From Genzyme
- DSEPC: 1,2-distearoyl-sn-glycero-3-ethyl-phosphocholin, from Avanti Polar Lipids

#### SOLVENTS:

- Perfluoro-n-hexane ( $C_6F_{14}$ ), from Fluka
- perfluoromethylcyclohexane (cyclo-methyl- $C_6F_{11}$ ), from Fluka
- perfluoro-n-heptane ( $C_7F_{16}$ ), from Fluka
- perfluoro-n-nonane ( $C_9F_{20}$ ), from Aldrich
- perfluorodecalin, from Aldrich
- Cyclohexane, from Fluka
- Cyclooctane, from Fluka
- n-Decane, from Fluka
- n-Octane, from Fluka
- meta xylène, from Fluka
- Diisopropyl cetone, from Fluka
- $CCl_4$ , from Fluka

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**LYOPROTECTANTS:**

- Mannose, from Fluka
- Glucose, from Fluka
- Sorbitol, from Fluka
- 5 Mannitol, from Fluka
- Maltose, from Fluka
- Dextran 6000, from Fluka
- Dextran 15000, from Fluka
- Dextran 40000, from Fluka
- 10 Inulin, from Fluka

**CHARACTERIZATION OF MICRODROPLETS AND MICROBUBBLES.**

The size distribution of the emulsions microdroplets has been determined:

- a) by means of a Coulter counter (Counter Mark II apparatus fitted with a  
15 30  $\mu\text{m}$  aperture with a measuring range of 0.7 to 20  $\mu\text{m}$ ), when the emulsion  
has been submitted to a washing step; 10  $\mu\text{l}$  of emulsion were diluted in 100 ml  
of saline at room temperature and allowed to equilibrate for 3 minutes prior to  
measurement.

- b) by means of a laser light scattering particle sizer (Malvern Mastersizer,  
20 dilution 200x, focal length 45 mm; standard presentation), if the emulsion has  
not been subjected to a washing step.

- The size distributions and volume concentrations of the microbubbles (after  
lyophilisation and reconstitution with an aqueous phase) were determined by  
using a Coulter Counter Mark II apparatus fitted with a 30  $\mu\text{m}$  aperture with a  
25 measuring range of 0.7 to 20  $\mu\text{m}$ . 50  $\mu\text{l}$  of microbubble samples were diluted in  
100 ml of saline at room temperature and allowed to equilibrate for 3 minutes  
prior to measurement.

**LYOPHILIZATION**

- The lyophilization methodology and apparatus were as follows. The  
30 emulsion (optionally after the washing step, if present) is first frozen at  $-45^{\circ}\text{C}$   
for 5 minutes and then freeze-dried (lyophilized) at room temperature at a  
pressure of 0.2 mbar, by using a Christ-Alpha 2-4 freeze-drier.

**Example 1 (preparations 1a-1n)**

- 35 DPPS, in a concentration of 1.0 mg/ml (0.1%), was added to about 10 ml of  
an aqueous solution containing mannitol (10% w/w), heated at  $65^{\circ}\text{C}$  for 15  
minutes and then cooled at room temperature ( $22^{\circ}\text{C}$ ). Perfluoroheptane (8%  
v/v) was added to this aqueous phase and emulsified in a beaker of about 4 cm  
diameter by using a high speed homogenizer (Polytron T3000, probe diameter

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of 3 cm) for 1 minute at the speed indicated in table 1. The resulting median diameter in volume ( $D_{V50}$ ) and a mean diameter in number ( $D_N$ ) of microdroplets of the emulsion are shown in table 1. The emulsion was then centrifuged (800-1200 rpm for 10 minutes, Sigma centrifuge 3K<sup>10</sup>) to eliminate the excess of the phospholipid and the separated pellets (microdroplets) were recovered and re-suspended in the same initial volume of a 10% mannitol aqueous solution.

The washed emulsion was then collected into a 100 ml balloon for lyophilization, frozen and then freeze-dried according to the above standard procedure. The lyophilized was then exposed to an atmosphere containing 35% of perfluoro-n-butane and 65% of nitrogen and then dispersed in a volume of water twice than the initial one by gentle hand shaking. The microbubble suspension obtained after reconstitution with distilled water was analyzed using a Coulter counter. The concentration of microbubbles in the obtained suspensions was of about  $1 \times 10^9$  particles per ml. The respective microbubbles median diameter in volume ( $D_{V50}$ ), mean diameter in volume ( $D_V$ ), mean diameter in number ( $D_N$ ) and the amount of microbubbles with diameter larger than 3  $\mu\text{m}$  are given in table 1. When more than one example has been performed at the same agitation speed, the values indicated in table 1 are referred to the mean calculated value of each parameter.

TABLE 1

Ex.	EMULSION			Gas-filled microbubbles				
	Agitation (rpm)	$D_{V50}$ ( $\mu\text{m}$ )	$D_N$ ( $\mu\text{m}$ )	$D_{V50}$ ( $\mu\text{m}$ )	$D_V$ ( $\mu\text{m}$ )	$D_N$ ( $\mu\text{m}$ )	$D_{V50}/D_N$	>3 $\mu\text{m}$ (%)
1a	8000	4.58	1.77	2.92	3.33	1.51	1.93	5.44
1b	9000	4.66	1.94	3.19	3.45	1.53	2.08	6.61
1c	10000	3.04	1.74	2.16	2.53	1.33	1.62	1.88
1d	11000	3.05	1.80	2.17	3.33	1.32	1.65	1.55
1e	12000	2.84	1.69	1.86	2.17	1.24	1.50	0.93
1f	12500	2.79	1.68	1.75	2.05	1.22	1.44	0.65
1g	14000	2.20	1.52	1.39	2.45	1.08	1.29	0.23
1h	14500	2.00	1.38	1.19	1.39	1.01	1.19	0.06
1i	15000	1.88	1.39	1.22	2.20	1.01	1.21	0.06
1j	15500	2.19	1.48	1.24	1.46	1.02	1.22	0.11
1k	16000	1.83	1.32	1.27	3.08	0.99	1.28	0.10
1l	17000	1.40	1.12	0.91	1.03	0.87	1.05	0.01

**Example 2 (preparations 2a-2j)**

The same procedure adopted for example 1 was followed, with the only difference that the phospholipid was a mixture of DPPS (20% w/w) and DSPC

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(80% w/w), the total amount of phospholipid remaining unchanged. The results are summarized in table 2.

**TABLE 2**

Ex.	EMULSION			Gas-filled microbubbles				
	Agitation (rpm)	D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> ( $\mu$ m)	D <sub>V</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> /D <sub>N</sub>	>3 $\mu$ m (%)
2a	6000	8.75	3.07	7.55	9.05	2.27	3.33	21.81
2b	10000	3.54	1.90	3.00	3.71	1.47	2.04	5.05
2c	12000	3.04	1.83	2.45	3.73	1.32	1.85	2.15
2d	12500	2.85	1.76	2.21	3.24	1.27	1.74	1.57
2e	13000	2.98	1.83	2.25	3.04	1.28	1.76	1.76
2f	13500	2.91	2.05	1.88	2.46	1.20	1.57	0.87
2g	14000	2.45	1.67	1.82	2.66	1.16	1.57	0.57
2h	14500	2.18	1.55	1.58	3.04	1.09	1.44	0.38
2i	15000	1.94	1.42	1.34	1.96	1.04	1.28	0.31
2j	16000	1.81	1.38	1.35	2.30	1.03	1.31	0.14

### 5 Example 3 (preparation 3a-3p)

The same procedure adopted for examples 2 was followed, with the only difference that the DPPS/DSPC ratio was varied, as reported in table 3. The results are summarized in table 3.

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Table 3

Ex.	DPPS / DSPC ratio	EMULSION			Gas-filled microbubbles			
		Agitation (rpm)	D <sub>V50</sub> (μm)	D <sub>N</sub> (μm)	D <sub>V50</sub> (μm)	D <sub>N</sub> (μm)	D <sub>V50</sub> / D <sub>N</sub>	>3μm (%)
3a	80/20	12000	2.44	1.54	1.68	1.19	1.41	0.48
3b	75/25	12000	2.53	1.66	1.73	1.18	1.47	0.62
3c	60/40	11000	3.53	1.86	2.75	1.45	1.90	4.00
3d	60/40	12000	2.62	1.60	1.78	1.21	1.47	0.72
3e	60/40	14000	2.36	1.60	1.59	1.13	1.41	0.36
3f	50/50	12000	2.81	1.68	2.28	1.30	1.75	2.05
3g	40/60	11000	3.00	1.72	2.44	1.32	1.85	2.31
3h	40/60	12000	2.88	1.75	2.07	1.27	1.63	1.45
3i	40/60	13000	2.61	1.69	1.76	1.16	1.52	0.57
3j	40/60	14000	2.06	1.43	1.41	1.07	1.31	0.23
3k	40/60	14500	2.39	1.67	1.64	1.15	1.43	0.49
3l	30/70	11000	3.12	1.75	2.64	1.37	1.93	2.76
3m	30/70	12000	3.08	1.81	2.38	1.34	1.78	2.45
3n	25/75	11000	3.15	1.85	2.46	1.31	1.88	2.15
3o	10/90	11000	3.72	2.26	3.14	1.47	2.13	4.60
3p	5/95	11000	4.53	2.23	4.08	1.54	2.65	6.35

**Example 4**

5 The same procedure adopted for example 2 was followed, with the only difference that the mixtures of DSPA and DPPS with different relative ratios were prepared. The results are summarized in table 4.

Table 4

Ex.	DSPA / DPPS ratio	Emulsion			Gas-filled microbubbles			
		Agitation (rpm)	D <sub>V50</sub> (μm)	D <sub>N</sub> (μm)	D <sub>V50</sub> (μm)	D <sub>N</sub> (μm)	D <sub>V50</sub> / D <sub>N</sub>	>3μm (%)
4a	25/75	12000	2.61	1.63	1.94	1.24	1.56	1.07
4b	50/50	11000	2.81	1.86	2.35	1.39	1.69	2.67
4c	50/50	12000	2.35	1.57	1.84	1.19	1.55	0.74
4d	75/25	12000	2.50	1.65	2.11	1.27	1.66	1.45

**Example 5 (preparations 5a-5i)**

10 The same procedure adopted for example 2 was followed, with the only difference that a 1/1 phospholipid mixture of DPPG and DSPC has been employed (total concentration 1.0 mg/ml) in admixture with 10% w/w (with respect to the total weight of phospholipid) of palmitic acid. The results are summarized in table 5.

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Table 5

Ex	EMULSION			Gas-filled microbubbles			
	Agitation (rpm)	D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> /D <sub>N</sub>	>3 $\mu$ m (%)
5a	6000	10.02	2.64	6.87	2.07	3.32	18.00
5b	8000	5.31	2.49	3.73	1.62	2.30	7.97
5c	9000	5.04	2.69	3.20	1.55	2.06	6.22
5d	10000	3.82	2.02	2.85	1.38	2.07	2.65
5e	10500	3.36	1.96	2.51	1.32	1.89	2.44
5f	11000	3.22	1.87	2.31	1.28	1.81	1.41
5g	12000	2.69	1.61	1.74	1.14	1.53	0.52
5h	13000	2.28	1.56	1.56	1.07	1.46	0.23
5i	14000	2.00	1.44	1.30	1.00	1.30	0.26

**Example 6**

The same procedure adopted for example 1 was followed, with the only difference that DSEPC was used as phospholipid and perfluorohexane was used as the organic solvent. The applied rotation speed was of 11000 rpm.

Dimensions, size distribution and percentage of microbubbles with diameter lower than 3 $\mu$ m were as follows.

D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> /D <sub>N</sub>	>3 $\mu$ m (%)
1.65	1.11	1.49	0.30

**Example 7 (preparations 7a-7i)**

Distilled water (10 ml) containing DPPS (10 mg) as phospholipid was heated at 70°C for 15 minutes and then cooled at room temperature. 0.8 ml of an organic solvent as specified in the following table 6 were emulsified in this aqueous phase using a high speed homogenizer (Polytron T3000) at 10000 rpm for 1 minute. The emulsion was added to 10 ml of a 15% dextran 15000 solution, frozen and lyophilized (0.2 mbar, 24 hours). After lyophilisation, air was introduced in the lyophiliser. The microbubble suspension obtained after reconstitution with distilled water was analysed using a Coulter counter. Table 6 summarizes the results in terms of dimensions and size distribution of microbubbles.

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TABLE 6

Ex.	Solvent	D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> /D <sub>N</sub>
7a	C <sub>6</sub> F <sub>14</sub>	2.77	1.44	1.92
7b	Cyclo-methyl-C <sub>6</sub> F <sub>11</sub>	2.24	1.30	1.72
7c	C <sub>7</sub> F <sub>16</sub>	2.48	1.40	1.77
7d	C <sub>9</sub> F <sub>20</sub>	2.46	1.36	1.81
7e	perfluorodecalin	3.76	1.52	2.47
7f	Cyclohexane	2.61	1.41	1.85
7g	Cyclooctane	2.43	1.35	1.80
7h	Decane	2.01	1.12	1.79
7i	Octane	2.87	0.96	2.99
7j	meta xylène	2.45	1.21	2.02
7k	Diisopropyl cetone	1.83	1.05	1.74
7l	CCl <sub>4</sub>	1.90	1.27	1.50

**Example 8**

5 The above example was repeated with the same methodology, by using perfluoro hexane as the organic solvent and different lyoprotecting agents at different concentrations as outlined in table 7. Table 7 summarizes the results in terms of dimensions and size distribution of microbubbles.

TABLE 7

Ex.	Lyoprotectant and concentration (w/w)	D <sub>V50</sub> ( $\mu$ m)	D <sub>N</sub> ( $\mu$ m)	D <sub>V50</sub> /D <sub>N</sub>
8a	Mannose 5%	4.35	1.90	2.29
8b	Glucose 5%	2.59	0.96	2.70
8c	Sorbitol 5%	3.84	1.40	2.74
8d	Mannitol 10%	2.22	1.22	1.82
8e	Mannitol 5%	2.24	1.21	1.85
8f	Mannitol 4%	2.54	1.45	1.75
8g	Maltose 5%	3.42	0.99	3.45
8h	Dextran 6000 7.5%	3.30	1.48	2.23
8j	Dextran 15000 5%	2.55	1.31	1.95
8k	Dextran 15000 7.5%	2.77	1.44	1.92
8i	Dextran 40000 7.5%	2.54	1.32	2.29
8l	Inulin 5%	3.58	1.43	2.70

10 **EXAMPLE 9 (preparations 9a-9e)**

Example 1 was repeated by emulsifying the mixture at a speed of 10000 rpm. In addition, the same example was repeated by adding different amounts of Pluronic F68 (a poloxamer corresponding to Poloxamer 188) into the aqueous phase prior to emulsification, as outlined in table 8. Table 8 shows the results of



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the comparative experiment, in terms of size distribution and conversion yield of the microbubbles. Conversion yield is given as the percentage number of gas-filled microbubbles formed upon reconstitution of the lyophilized matrix with respect to the number of microdroplets measured in the emulsion.

5 **TABLE 8**

Example	Pluronic* (mg/ml)	D <sub>V50</sub>	D <sub>N</sub>	D <sub>V50</sub> /D <sub>N</sub>	Conversion yield (%)
9a	0	2.42	1.38	1.75	28.0
9b	0.25	4.64	1.97	2.36	18.8
9c	0.5	13.85	1.38	10.04	7.3
9d	1.0	12.59	1.49	8.45	3.2
9e	2.0	15.80	1.23	12.85	0.5

\*Concentration referred to the volume of aqueous phase

The above results show that with a concentration of poloxamer corresponding to half the concentration of the phospholipid, both conversion yields and size distribution of microbubbles are negatively affected.

10

**EXAMPLE 10 (preparations 10a-10d)**

Example 9 was repeated, but instead of adding Pluronic F68 to the aqueous phase, different amounts of cholesterol (from Fluka) were added to the organic phase, prior to emulsification, as outlined in table 9. Table 9 shows the results of the comparative experiment, in terms of size distribution and conversion yield (from the microdroplets of the emulsion) of the microbubbles.

15

**TABLE 9**

Example	Cholesterol* (mg/ml)	D <sub>V50</sub>	D <sub>N</sub>	D <sub>V50</sub> /D <sub>N</sub>	Conversion yield (%)
10a	0	2.42	1.38	1.75	28.0
10b	0.10	3.79	1.31	2.89	17.8
10c	0.25	1.35	1.05	1.28	5.7
10d	0.50	14.02	1.70	8.25	0.8

\*Concentration referred to the volume of the aqueous phase

20

The above results show that with a concentration of 0.050% (w/w) of cholesterol in the aqueous phase, both conversion yield and size distribution of microbubbles are highly negatively affected. A concentration of 0.025%, while it may provide acceptable dimensions and size distribution of microbubbles, still results in a rather low conversion yield.

25

**Example 11.**

Distilled water (30 ml) containing 60 mg of DPPS and 3g of mannitol was heated to 70°C during 15 minutes then cooled to room temperature.

Perfluoroheptane was emulsified in this aqueous phase using a high speed homogenizer (Polytron<sup>®</sup>, 12500rpm, 1 minute).

The resulting emulsion showed a median diameter in volume ( $D_{V50}$ ) of 2.3 $\mu$ m and a mean diameter in number ( $D_N$ ) of 2.0  $\mu$ m as determined with a Malvern Mastersizer.

The emulsion was washed once by centrifugation, resuspended in 30ml of a 10% solution of mannitol in distilled water and then divided in three portions (3x10ml).

The first portion (A) was used as such for the subsequent lyophilization step. The second portion (B) was collected into a syringe and hand-injected through a 5 $\mu$ m Nuclepore<sup>®</sup> filter (47mm - Polycarbonate).

The third portion (C) was filtered through a 3 $\mu$ m Nuclepore<sup>®</sup> filter (47mm - Polycarbonate) with the same method.

The emulsions were frozen in 100ml balloon (-45°C for 5 minutes) then freeze dried (0.2 mBar, for 72 hours).

Atmospheric pressure was restored by introducing a 35/65 mixture of C<sub>4</sub>F<sub>10</sub> and air. The respective lyophilisates were dissolved in distilled water (10ml). The so obtained microbubbles suspensions were analysed using a Coulter counter and the results are reported in the following table

	$D_{V50}$ ( $\mu$ m)	$D_N$ ( $\mu$ m)	$D_{V50}/D_N$
Part A	1.71	1.12	1.53
Part B	1.65	1.12	1.47
Part C	1.57	1.09	1.44

As shown by the above results, the additional filtration step allows to further reduce the dimension of the microbubbles and to reduce the respective size distribution.

**Example 12**

Distilled water (10 ml) containing 10 mg of DPPS and 1 g of mannitol was heated to 70°C during 15 minutes then cooled to room temperature. DPPE-MPB(1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] Na salt - Avanti Polar Lipids) was added (4.8%

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by weight ~ 0.5mg). This phospholipid was dispersed in the aqueous phase using a ultrasound bath (Branson 1210 - 3 minutes).

Perfluoroheptane (0.8ml from Fluka) was emulsified in this aqueous phase (cooled with a ice bath) using a high speed homogenizer (Polytron® T3000, 5 15000rpm, 1 minute).

The resulting emulsion showed a median diameter in volume ( $D_{V50}$ ) of 2.3µm and a mean diameter in number ( $D_N$ ) of 2.1µm as determined with a Malvern Mastersizer.

The emulsion was washed twice by centrifugation then resuspended in 9.5 10 ml of a 10% solution of mannitol in distilled water. The washed emulsion was frozen (-45°C, 5 minutes) then freeze dried (under 0.2 mBar, for 24 hours).

Atmospheric pressure was restored by introducing a 35/65 mixture of C<sub>4</sub>F<sub>10</sub> and air. The lyophilisate was dissolved in distilled water (20 ml), microbubbles were washed once by centrifugation and then redispersed in 4ml of an EDTA 15 containing phosphate buffer saline (molar composition: 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, 10 mM EDTA), containing 3.4 mg of thioacetylated avidin, 400 µl of a hydroxylamine solution (13.92 mg in PBS 50 mM, pH: 7.5) were added to deprotect the thiol group of the thioacetylated avidin.

The suspension was stirred by inversion on a disk rotator (Fisher Scientific) 20 for 2 hours. Then 150 µl of NaOH 1N were added.

The so obtained avidin-labelled microbubbles were washed twice with PBS by centrifugation (10000 rpm, 10 minutes, Sigma centrifuge 3K<sup>10</sup>). The microbubbles suspension obtained was analysed using a Coulter counter showing a  $D_{V50}$  diameter of 1.6 µm and a  $D_N$  of 1.2 µm.

25 The efficacy of targeted microbubbles composition was tested both in vitro and in vivo.

#### In vitro experiment:

To test the effective bonding of acetylated avidin to the surface of the microbubbles, two sets of fibrin containing wells were prepared. In the first set, 30 only a fibrin surface was present. In the second set, the fibrin was pre-treated with a biotin-labelled antifibrin peptide (DX-278, disclosed WO 02/055544). Microbubble suspensions prepared as above were added to the wells ( $5 \times 10^6$  microbubbles/well). After 2 hours of incubation (upside down) and several washings, the fibrin surfaces in the two set of wells were observed by means of 35 an optical microscope. While essentially no microbubble could be observed in the wells without the biotinylated antifibrin peptide, a massive coverage of microbubbles was observed in the biotinylated antifibrin peptide containing wells.

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In vivo experiment: A thrombus was formed in the abdominal aorta of two rabbits by the  $\text{FeCl}_3$  method (Lockyer et al, 1999, Journal of Cardiovascular Pharmacology, vol 33, pp 718-725).

- 5 Echo Imaging was performed with an ATL HDI 5000 ultrasound machine (pulse Inversion mode – L7-4 probe – MI: 0.07).

A biotinylated antibody (CD41 specific for the GPIIB/IIIA receptor of activated platelets) was then injected intravenously to the two rabbit.

- 10 After 30 minutes, the microbubble suspension comprising avidin-labelled microbubbles was injected intravenously ( $1 \times 10^9$  microbubbles/ml) in the first rabbit. Fifteen minutes after the injection, a strong opacification of the thrombus was observed for the suspension. This opacification was still visible after at least one hour from the injection.

- 15 The same amount of the microbubble suspension without avidin-labelled microbubbles was injected intravenously in the second rabbit. Only a light opacification of the thrombus was observed.

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**CLAIMS**

1. Method for preparing a lyophilized matrix which, upon contact with an aqueous carrier liquid and a gas, is reconstitutable into a suspension of gas-filled microbubbles stabilized essentially by a phospholipid, said method comprising the steps of:

- a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) a phospholipid and iv) a lyoprotecting agent;
- b) lyophilizing said emulsified mixture, to obtain a lyophilized matrix comprising said phospholipid.

2. Method for preparing an injectable contrast agent comprising a liquid aqueous suspension of gas-filled microbubbles stabilized essentially by a phospholipid, which comprises the steps of:

- a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) a phospholipid and iv) a lyoprotecting agent;
- b) lyophilizing said emulsion, to obtain a lyophilized matrix comprising said phospholipid;
- c) contacting said lyophilized matrix with a biocompatible gas;
- d) reconstituting said lyophilized matrix by dissolving it into an aqueous carrier liquid, to obtain a suspension of gas-filled microbubbles stabilized essentially by said phospholipid.

3. Method according to claim 1 or 2 wherein the step a) of preparing the emulsion comprises the following steps:

- a1) preparing a solution by dispersing the phospholipid and the lyoprotective agent in the aqueous medium;
- a2) admixing the obtained solution with the organic solvent;
- a3) submitting the mixture to controlled agitation, to obtain an emulsion.

4. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of less than 10 g/l.

5. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 1.0 g/l or lower.

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6. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 0.2 g/l or lower.

5 7. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of about 0.01 g/l or lower.

8. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 0.001 g/l or lower.

10

9. Method according to any of the preceding claims, wherein the organic solvent is selected among branched or linear alkanes, alkenes, cyclo-alkanes, aromatic hydrocarbons, alkyl ethers, ketones, halogenated hydrocarbons, perfluorinated hydrocarbons and mixtures thereof.

15

10. Method according to claim 9 wherein the solvent is selected among pentane, hexane, heptane, octane, nonane, decane, 1-pentene, 2-pentene, 1-octene, cyclopentane, cyclohexane, cyclooctane, 1-methyl-cyclohexane, benzene, toluene, ethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene, di-butyl ether and di-isopropylketone, chloroform, carbon tetrachloride, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane (enflurane), 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane), tetrachloro-1,1-difluoroethane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorononane, perfluorobenzene, perfluorodecalin and mixtures thereof.

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25

11. Method according to any of the preceding claims, wherein the amount of organic solvent is from about 1% to about 50% by volume with respect to the amount water.

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12. Method according to any of the preceding claims wherein the lyoprotecting agent is selected among carbohydrates, sugar alcohols, polyglycols and mixtures thereof.

35

13. Method according to claim 12 wherein the lyoprotecting agent is selected among glucose, galactose, fructose, sucrose, trehalose, maltose, lactose, amylose, amylopectin, cyclodextrins, dextran, inuline, soluble starch, hydroxyethyl starch (HES), erythritol, mannitol, sorbitol, polyethyleneglycols and mixtures thereof.

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**14.** Method according to claim 12 or 13 wherein the amount of lyoprotecting agent from about 1% to about 25% by weight with respect to the weight of water.

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**15.** Method according to any of claims 1, 2 or 3 wherein the phospholipid is selected among dilauryloyl-phosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DMPC"), dipalmitoyl-phosphatidylcholine ("DPPC"), diarachidoylphosphatidylcholine ("DAPC"), distearoyl-phosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoylphosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoylphosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoylphosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl-phosphatidylcholine ("SPPC"), dioleoylphosphatidylcholine ("DOPC"), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dilauryloyl-phosphatidylglycerol ("DLPG") and its alkali metal salts, diarachidoylphosphatidylglycerol ("DAPG") and its alkali metal salts, dimyristoylphosphatidylglycerol ("DMPG") and its alkali metal salts, dipalmitoyl-phosphatidylglycerol ("DPPG") and its alkali metal salts, distearoylphosphatidylglycerol ("DSPG") and its alkali metal salts, dioleoylphosphatidylglycerol ("DOPG") and its alkali metal salts, dimyristoyl phosphatidic acid ("DMPA"), dipalmitoyl phosphatidic acid ("DPPA"), distearoyl phosphatidic acid ("DSPA"), diarachidoyl phosphatidic acid ("DAPA"), dimyristoyl phosphatidyl-ethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), distearoyl phosphatidyl-ethanolamine ("DSPE"), dimyristoyl phosphatidylserine ("DMPS"), diarachidoyl phosphatidylserine ("DAPS"), dipalmitoyl phosphatidylserine ("DPPS"), distearoylphosphatidylserine ("DSPS"), dioleoylphosphatidylserine ("DOPS"), dipalmitoyl sphingomyelin ("DPSP"), distearoyl sphingomyelin ("DSSP") and mixtures thereof.

30

**16.** Method according to claim 15 wherein the phospholipid is a mixture of at least one neutral phospholipid and one phospholipid with an overall net charge.

**17.** Method according to claim 1, 2, 3 or 13, wherein the amount of phospholipid is from about 0.005% to about 1.0% by weight with respect to the total weight of the emulsified mixture.

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18. Method according to claim 17 wherein the amount of of phospholipid is from 0.01% to 1.0% by weight with respect to the total weight of the emulsified mixture.

5 19. Method according to claim 1, 2 or 3 wherein the phospholipid includes a targeting ligand or a protective reactive group capable of reacting with a targeting ligand.

10 20. Method according to any of claims 1, 2, 3, 15 or 16 wherein the emulsion further contains a fatty acid.

21. Method according to claim 1 or 2 wherein the aqueous-organic emulsion of step a) is subjected to a washing step before the lyophilizing step b).

15 22. Method according to claim 1 or 2 wherein the aqueous-organic emulsion of step a) is subjected to a microfiltration step before the lyophilizing step b).

20 23. Method according to claim 2 or 3 wherein the biocompatible gas is selected among air; nitrogen; oxygen; carbon dioxide; hydrogen; nitrous oxide; inert gases; a low molecular weight hydrocarbon, including a (C<sub>1</sub>-C<sub>7</sub>) alkane, a (C<sub>4</sub>-C<sub>7</sub>) cycloalkane, a (C<sub>2</sub>-C<sub>7</sub>) alkene and a (C<sub>2</sub>-C<sub>7</sub>) alkyne; an ether; a ketone; an ester; a halogenated (C<sub>1</sub>-C<sub>7</sub>) hydrocarbon, ketone or ether; or a mixture of any of the foregoing.

25 24. Method according to claim 23 wherein the halogenated hydrocarbon gas is selected among bromochlorodifluoro-methane, chlorodifluoromethane, dichlorodifluoro-methane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, dichlorotetrafluoroethane and mixtures thereof.

30 25. Method according to claim 23 wherein the halogenated hydrocarbon gas is a perfluorinated hydrocarbon.

35 26. Method according to claim 25 wherein the perfluorinated hydrocarbon gas is perfluoromethane, perfluoroethane, a perfluoropropane, a perfluorobutane, a perfluoropentane, a perfluorohexane, a perfluoroheptane; perfluoropropene, a perfluorobutene, perfluorobutadiene, perfluorobut-2-yne, perfluorocyclobutane, perfluoromethylcyclobutane, a perfluorodimethylcyclobutane, a perfluorotrimethylcyclo-butane,



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perfluorocyclopentane, perfluoromethylcyclopentane, a perfluorodimethylcyclopentane, perfluorocyclohexane, perfluoromethylcyclohexane, perfluoromethylcyclohexane and mixtures thereof.

5     **27.** Injectable aqueous suspension of microbubbles filled with a biocompatible gas and comprising a stabilizing layer of a phospholipid, wherein said microbubbles have a number mean diameter ( $D_N$ ) of less than  $1.70\ \mu\text{m}$  and a volume median diameter ( $D_{V50}$ ) such that the  $D_{V50}/D_N$  ratio is of about 2.00 or lower.

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**28.** Aqueous suspension according to claim 27 wherein said microbubbles have a  $D_N$  value of  $1.60\ \mu\text{m}$  or lower, preferably of  $1.50\ \mu\text{m}$  or lower, more preferably of  $1.30\ \mu\text{m}$  or lower.

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**29.** Aqueous suspension according to claim 27 wherein said microbubbles have a  $D_{V50}/D_N$  ratio of about 1.80 or lower, preferably of about 1.60 or lower, more preferably of about 1.50 or lower.

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**30.** Contrast agent for use in diagnostic imaging comprising an aqueous suspension according to any of the claims 27 to 29.

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**31.** Method for diagnostic imaging comprising administering a contrast-enhancing amount of an aqueous suspension according to any of the claims 27 to 29.

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**Abstract**

Method for preparing a lyophilized matrix and, upon reconstitution of the same, a respective injectable contrast agent comprising a liquid aqueous suspension of gas-filled microbubbles stabilized essentially by a phospholipid.

The method comprises preparing an emulsion from an aqueous medium, a phospholipid and a water immiscible organic solvent. The emulsion is then freeze-dried and subsequently reconstituted in an aqueous suspension of gas-filled microbubbles.

The method allows to obtain suspension comprising microbubbles having a relatively small diameter and a narrow size distribution.

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